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Screening for Toxic Effects on Interspecies Interactions: A Mechanistic or an Empirical Approach?

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Abstract. The use of empirical and mechanistic approaches are possible in the development of tests to screen for a substance's potential to affect interspecies interactions. The advantages and disadvantages of the two approaches are discussed. An experimental study is presented, in which an empirical and a mechanistic screening test for effects on exploitative competition between bacterial species were established and perturbed with nalidixic acid. Comparison of test results indicates that the mechanistic test was faster, cheaper, more sensitive, and more quantitative. The empirical test attained similar sensitivity and quantification only if the dynamics of the competition event was continuously monitored; requiring even greater cost and time.

The number of man-made chemicals present in the environment is estimated at greater than two million, with approximately 250,000 added each year (Maki 1979). Many of these substances may have adverse effects on natural communities via direct toxicity to the constituent organisms or alterations in the interactions between these organisms. In 1976, the Toxic Substance Control Act (P.L. 94-469) legislated a need to develop adequate test protocols to identify which substances have a potential for adverse effects on natural communities and to regulate their use (Train 1978). Currently, screening tests are available to address the potential of substances which directly affect single species populations. Selected test species are exposed to a suspected toxicant, and alteration in population level parameters (*i.e.*, mortality, fecundity, growth rate, *etc.*) are used to identify effect concentrations. These tests may not be fully perfected, but do have a firm conceptual and developmental basis. On the other hand, screening tests to address the potential for effects on interspecies interactions are still in the early theoretical phase of development. This paper will examine two possible approaches to the development of these interspecies

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interaction screening tests and present experimental data to illustrate their relative sensitivities.

The Approaches

A number of investigators have proposed screening tests for effects on interspecies interactions by using an empirical approach (Fisher *et al.* 1974; Kricher *et al.* 1979; Mosser *et al.* 1972; Crow and Taub 1979; Kersting 1975; Taub 1976; Kwasnik 1977; Sullivan and Atchison 1978; Sullivan *et al.* 1978). In these studies, systems are established which contain two or more interacting species (*i.e.*, competitors, predator(s) and prey, or some combination of the two). Under unperturbed conditions, the interactions result in a specific sequence of events, culminating in a standard outcome, which serves as the control. These outcomes may include competitive exclusion, competitive coexistence, predator-prey coexsistence, or predator-prey extinction. A substance is identified as having an effect on the interaction if it caused a departure from the control outcome. For example, if a substance causes a reversal as to which competitor is eliminated in the test system, it would be identified as harmful to that interaction. This approach is attractive, because it gives a clear cut indication of an effect on an interaction (*i.e.*, an actual alteration in the interaction is observed). However, it has a potential serious flaw in its use as a screening tool. It may identify substances as harmless, which in fact will affect interactions in some natural communities. This possibility arises, because of the dependence of this empirical approach on a specific species combination. Species combinations found in natural communities may prove more sensitive to the substance being tested than the combination established in the screening test. Assessing the relative sensitivity of the test system will prove difficult, because of the large number of possible combinations of species and the added complication of interfering interactions. It is possible that a mechanistic approach would be more appropriate in the development of these interaction screening tests. In this approach, factors which control a species' ability to be successful in an interaction are identified; *i.e.*, a competitor's ability to use limited resources, a predator's ability to capture prey, or a prey's ability to escape predation. It is the state of these factors, measured on single species populations of test organisms, which serve as the test parameters. The normal state of these factors is experimentally determined under unperturbed conditions and serves as the control. If a suspected toxic substance alters the values of these factors from the control values, it is flagged as being potentially harmful to actual interactions between species. The potential of the toxicant to affect an interaction is implied from a measured effect on a single species' ability to interact; no interaction event is established or observed. For example, a species' success in exploitative competition is controlled by how well it can grow and reproduce under conditions of resource limitation. If a suspected toxicant reduces a competitior's ability to grow and/or reproduce under these limited conditions, then it affects the species' competitive ability and, therefore, would be identified as being potentially harmful to the outcome of an actual exploitative interaction event. This mechanistic approach is attractive as a screening test, because of its low probability of clearing harmful substances. However, the approach does have the potential to err on the side of overprotection. Some substances screened as potentially harmful may not affect the interactions in any real community. This possibility arises, because the screen is not set up to predict whether a substance will actually affect the interaction between any particular set of species. No interaction situation is established, and no alteration in a particular situation is observed. The test only indicates that species have suffered reduced interaction abilities and that the potential for effects in real interaction situations exists. Whether effects are actually seen in a natural community is dependent upon the species composition of the community and the degree to which each of the component species is affected. In communities in which the interacting species are affected to different degrees—*i.e.*, one competitor's ability to use limited resources is reduced more than the other competitor's abilities, or a prey's ability to escape predation is reduced more than a predator's ability to catch it—an effect will be seen. In communities in which the interacting species are affected to the same degree—*i.e.*, all competitors suffer the same reduced ability to use limited resources, or a prey's ability to escape predation is reduced the same as a predator's ability to capture it—no effect will be seen.

The Experiment

A comparative study was carried out to illustrate the methodologies and to compare the relative sensitivities of these two approaches. A mechanistic and an empirical test were established to screen for the potential of the toxic substance, nalidixic acid, to affect exploitative competition for limited tryptophan between two strains of the bacterium, *Escherichia coli*. Exploitative competition was chosen as the interaction type due to its common occurrence between micro-organisms in many natural communities and, perhaps more importantly, to the theoretical elucidation and experimental verification of the factors which control the outcome of this form of competition (Monod 1942; Novick and Szilard 1950; Titman 1976; Hsu *et al.* 1977; Hansen and Hubbell 1980). It was the identification of these factors which permitted the development of a mechanistic approach. In a purely exploitative competition situation, a species' competitive ability is dependent upon how rapidly its population can increase at low concentrations of limiting resources. The species which can grow the fastest on the lowest concentrations of the limiting resource will be competitively superior. This relationship is quantified as the ratio, Ks(D/Vmax), where Vmax is the species' maximum growth rate, Ks is the concentration of the limiting resource which allows half maximal growth and D is the species' death rate. For a particular limiting resource, the species with the lowest ratio is competitively superior. Any perturbation which alters one or more of these controlling factors, may alter the ratio and, consequently affect a species' competitive ability. Positive or negative changes in competitive ability could prove equally deleterious to a natural community. As long as species are differentially affected, both cases can lead to competitive exclusions and resulting community simplicity. Strains of *E. coli* were chosen as the test organisms due to their short generation times and the availability of tryptophan auxotrophs. Generation times of from one to several hr, depending upon resource availability, facilitate rapid analysis of outcomes in both the mechanistic and empirical systems. This is especially important in the case of an empirical test in which competitor abilities are very similar; outcomes may require generations. Tryptophan auxotrophs are necessary, to insure that competition for the limited tryptophan resource is occurring in the test system. If a species can manufacture tryptophan, competition may occur for some other unidentified limited resource, or not at all. In either case, the power of the screening test would be reduced or eliminated.

Materials and Methods

The test organisms were two F⁻ clones of *E. coli*, strain C-8. Both cultures were auxotrophic for tryptophan. One culture was resistant to the metabolic inhibitor nalidixic acid, but sensitive to spectinomysin (Nal^r Spec^s); the other culture had the reverse pattern of resistance (Nal^s Spec^r). The experimental conditions were established so that tryptophan was the limiting resource, and competition would occur for its acquisition. The mechanistic screening test was a measure of the ability of each strain to compete exploitatively for limited amounts of tryptophan. Two of the controlling factors, Ks and Vmax, were used as the test parameters. Base-line values of these test parameters were determined for each strain, under controlled physical and chemical conditions. Toxicant-added values were determined under identical test conditions. Differences between base-line and toxicant-added values were used as evidence that nalidixic acid had the potential to affect exploitative competition. The third controlling factor, D, was not included as a test parameter, because of the difficulty in identifying its component parts (*i.e.*, starvation, predation, senescence, *etc.*) and in estimating their relative importance. Inclusion of D is not essential for the operation of the screening test. However, depending upon the relative sensitivity of D to toxicant addition, its use could increase the overall sensitivity of the approach.

Values for Vmax and Ks were determined in batch culture for each of the test cultures under conditions of tryptophan limitation. A series of 500 ml flasks containing different concentrations of tryptophan in 100 ml of a minimal salts medium were incubated at 34°C in a constant temperature water bath equipped with a shaker, operating at 60 r.p.m. Log phase cells were inoculated into each flask at 10^3 cells/ml. For eight hours, at one hour intervals, samples were removed for enumeration of bacteria by standard plate count methods. The small inoculum and the short duration of the test insured that no significant reduction in batch tryptophan concentration occurred during the genera-

tion of the test parameters. A regression of ln (cells/ml) against time gave growth rate of the test species in log phase for each concentration of tryptophan in the culture medium. From the assembled growth rate and concentration data, estimates of Ks and Vmax were made, using a Lineweaver-Burk plot of 1/growth rate versus 1/tryptophan concentration.

The values of each test culture's growth parameters, Vmax and Ks, in the presence of nalidixic acid were determined using the same general procedures as in base-line determinations, except for the addition of the toxicant. For each toxicant concentration tested, a series of flasks containing different amounts of tryptophan were established, spiked with the toxicant, inoculated with bacteria, incubated, shaken, sampled and counted as before. Estimation of Ks and Vmax was made for each test culture at each toxicant concentration.

The empirical screening test consisted of the establishment of a two strain situation in which both strains exploitatively competed for limited tryptophan. The outcome of this competition situation, either competitive exclusion or co-existence, was observed and served as the test parameter. A base-line value of the outcome was determined under controlled physical and chemical conditions. Toxicant-added values were determined under identical conditions. Differences between the base-line and toxicant-added outcomes were taken as evidence that nalidixic acid had the potential to affect exploitative competition. In the empirical test, the base line competition situation was established in replicated (3) continuous-flow chemostats (VirTis). Log phase populations of the two test strains were aseptically inoculated into the sterile chemostats, containing 200 ml of culture medium and were incubated at 34°C in a constant temperature water bath, equipped with a shaker operating at 60 r.p.m. The culture medium was established at 5 mg/l of tryptophan and excess amounts of all other essential nutrients to insure that tryptophan was the limiting resource. The replacement rate of the culture medium was held at 7.5 ml/hr to limit cell production. The growth of each strain was monitored by sterile withdrawal, serial dilution, and replicate plating of samples. Sequential serial dilution was necessary to insure that accurate plate counts could be made, regardless of the cell density of the strain at the time of sampling (densities ranged over four to six orders of magnitude in the culture). Each strain was counted on two separate series of plates, each treated with an inhibitor to which one strain was resistant and the other sensitive; taking advantage of their differential sensitivities to nalidixic acid and spectinomysin. The sequence and the outcome of the base-line interaction event were observed in the replicated chemostat systems.

The competitive outcomes, in the presence of nalidixic acid, were determined by the same general procedure as the base-line determinations, except for the addition of one of the toxicant concentrations into the culture medium. Therefore, the continuous replacement of both nutrients and toxicant occurred at a rate of 7.5 ml/hr. For each toxicant concentration tested, a replicated (3) set of chemostats was established, inoculated with the two bacterial strains, shaken, sampled, and counted as before. The sequence and the outcome of these toxicant-added interaction events were observed in the replicated chemostat systems.

Results

The results from the mechanistic test are presented in Table 1. The values of the two test parameters, Ks and Vmax, and their ratio, Ks/Vmax, are given for the base-line and toxicant-added cases. At 0.2 μg/ml of nalidixic acid, the test parameters of both test strains remained unchanged. At 0.3 μg/ml, strain Nal^s experienced a decreased Vmax and consequently an increased Ks/Vmax; strain Nal^r was unaffected. At 0.5 μg/ml, strain Nal^s experienced a decreased Vmax and Ks and consequently an increased Ks/Vmax; strain Nal^r was still unaffected.

The results from the empirical test are presented in Figure 1. For the base-line and toxicant-added cases, the population dynamics of both strains and the outcomes of the compeition events are shown. In the base-line situation, strain Nal^s was competitively superior; eliminating strain Nal^r in 87 hr (Figure 1a). At 0.2 μg/ml, the results remained unchanged (Figure 1b). At 0.3

Table 1. Results of the mechanistic screening test. Base-line and toxicant-added values of the test parameters

Strain	Nalidixic acid added (μg/ml)	Ks (g/l)	Vmax (per hr)	Ks/Vmax
Nal^r	0	1.6×10^{-6}	0.68	2.35×10^{-6}
Nal^s		1.6×10^{-6}	0.96	1.67×10^{-6}
Nal^r	0.2	1.6×10^{-6}	0.68	2.35×10^{-6}
Nal^s		1.6×10^{-6}	0.96	1.67×10^{-6}
Nal^r	0.3	1.6×10^{-6}	0.68	2.35×10^{-6}
Nal^s		1.6×10^{-6}	0.82	1.95×10^{-6}
Nal^r	0.5	1.6×10^{-6}	0.68	2.35×10^{-6}
Nal^s		0.9×10^{-6}	0.40	2.25×10^{-6}

μg/ml, strain Nal^s was still competitively superior; however elimination of strain Nal^r was extended to 110 hr (Figure 1c). At 0.5 μg/ml, coexistence of strains Nal^s and Nal^r occurred for 130 hr (Figure 1d.)

Conclusions

1. Both the mechanistic and empirical approaches achieved the same level of sensitivity. In the mechanistic test, the lowest effect concentration of nalidixic acid was 0.3 μg/ml. At this concentration, the maximum growth rate, Vmax, of test strain Nal^s was decreased, resulting in a predicted reduction of competitive ability. In the empirical test, the identification of the low effect concentration was dependent upon the test criterion used. If an altered outcome in the competition event was the criterion, 0.5 μg/ml was the low effect level; the outcome was shifted from competitive exclusion to coexistence. If altered population dynamics was the criterion, 0.3 μg/ml was identified; the competitive exclusion of Nal^r still occurred, but its time to extinction increased. Therefore, the empirical test gains its greatest sensitivity only when a continuous analysis of each competitor's population density was made.

2. In order to gain its highest sensitivity, the empirical approach required longer analysis time and the use of more sophisticated techniques. Both approaches required the measurement of population densities of the test organisms. However, the mechanistic test only monitors the densities of single species populations, whereas the empirical test separately monitors the densities of each of the species contained in the multi-species test system. The separation of the system into its component species and their enumeration is considerably more difficult than the simple enumeration of a single species culture.

3. The mechanistic approach generated meaningful screening results more rapidly. The test produced values of its test parameters in eight hr. The empirical test ran for up to five days in order to produce definite outcomes to the competition events.

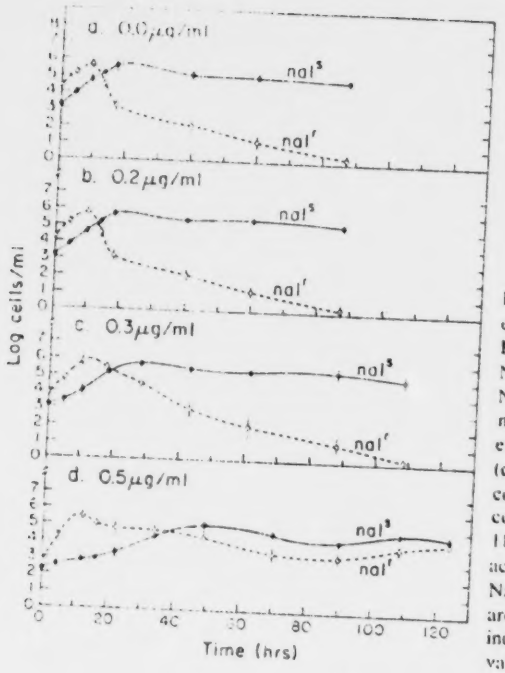

Fig. 1. Results from the empirical screening test. (a) Base-line competition event: Nal^s competitively eliminates Nal^r in 87 hr. (b) 0.2 μg/ml of nalidixic acid competition event: same results as base-line. (c) 0.3 μg/ml of nalidixic acid competition event: Nal^s competitively eliminates Nal^r in 110 hr. (d) 0.5 μg/ml of nalidixic acid competition event: Nal^s and Nal^r coexist for 130 hr. Bars around points in all experiments indicate ranges of three replicate values

4. The results generated in the mechanistic approach were more quantitative. For each concentration of nalidixic acid, the mechanistic test gave precise values for each of the test parameters. The percent deviation from base-line values, caused by each toxicant concentration, could be calculated and quantitatively fitted to a dose-response curve. These data could be used to establish regulatory standards; perhaps requiring a certain deviation to occur before classifying a substance as harmful. On the other hand, the results generated in the empirical test tended to be more qualitative. For the cases in which the outcome of the competition event was the test criterion, either effect or no-effect results were possible. These data are not appropriate for fitting to a dose-response curve. For the cases in which the dynamics of the competition event was the test criterion, population densities were measured over the course of the experiment. These data are more appropriate to dose-response curve fitting. However, they required accurate measurements of diverging population densities over the course of the competition event. In order to differentiate similar effect levels, more frequent measurements would be necessary; resulting in increased costs in terms of time and manpower.

5. Presently, the empirical approach can be applied to a broader range of interspecies interactions. Depending upon the availability of test organisms and the establishment of proper test conditions, modelled interaction events are possible for most interaction types. On the other hand, the range of the mechanistic approach is more limited; being applicable only to those interac-

tions whose underlying machanisms have been identified and experimentally proven. Due to the large amount of current interest, the number of interaction types meeting these requirements should make the mechanistic approach applicable to many more interaction types.

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